



# A novel mitochondrial carrier protein Mme1 acts as a yeast mitochondrial magnesium exporter

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## ABSTRACT

The homeostasis of magnesium (Mg<sup>2+</sup>), an abundant divalent cation indispensable for many biological processes including mitochondrial functions, is underexplored. Previously, two mitochondrial Mg<sup>2+</sup> importers, Mrs2 and Lpe10, were characterized for mitochondrial Mg<sup>2+</sup> uptake. We now show that mitochondrial Mg<sup>2+</sup> homeostasis is accurately controlled through the combined effects of previously known importers and a novel exporter, Mme1 (mitochondrial magnesium exporter 1). Mme1 belongs to the mitochondrial carrier family and was isolated for its mutation that is able to suppress the *mrs2Δ* respiration defect. Deletion of *MME1* significantly increased steady-state mitochondrial Mg<sup>2+</sup> concentration, while overexpression decreased it. Measurements of Mg<sup>2+</sup> exit from proteoliposomes reconstituted with purified Mme1 provided definite evidence for Mme1 as an Mg<sup>2+</sup> exporter. Our studies identified, for the first time, a mitochondrial Mg<sup>2+</sup> exporter that works together with mitochondrial importers to ensure the precise control of mitochondrial Mg<sup>2+</sup> homeostasis.

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## 1. Introduction

Magnesium (Mg<sup>2+</sup>) is a major intracellular divalent cation in living cells. As a critical co-factor for hundreds of enzymes, Mg<sup>2+</sup> has been known to participate in over 300 metabolic reactions, including but not limited to energy production, ion metabolism, and cell cycle regulation [1]. Mg<sup>2+</sup> also plays an important role in sustaining genomic stability and preventing carcinogenicity [2]. Therefore, it is quite apprehensible that alterations in Mg<sup>2+</sup> metabolism would correlate to many important human diseases [3], including metabolic syndromes such as type 2 diabetes mellitus and hypertension [4]. In higher plants, Mg<sup>2+</sup> also plays a critical role in regulating the chloroplast mRNA stability [5].

*Saccharomyces cerevisiae* has been proved to be an excellent model organism for the study of many fundamental biological processes, including the mechanism of Mg<sup>2+</sup> transport. Up to now, three types

of Mg<sup>2+</sup> transporters have been identified in yeast—the Alr Mg<sup>2+</sup> transport system in the cell plasma membrane, the Mnr2 Mg<sup>2+</sup> transporter in the vacuolar membrane, and the Mrs2/Lpe10 Mg<sup>2+</sup> transporter in the mitochondrial inner membrane.

The two Alr proteins (Alr1 and Alr2) are orthologues of the bacterial CorA transporter, sharing with the latter a highly conserved GMN motif [6]. Both Alr1 and Alr2 localize in the plasma membrane and function as Mg<sup>2+</sup> uptake [6,7].

In vacuoles, storage sites for Mg<sup>2+</sup> within yeast cells, a Mg<sup>2+</sup>/H<sup>+</sup> exchange mechanism was discovered to drive Mg<sup>2+</sup> entry into the organelles [8]. On the other hand, Mnr2 (manganese resistance 2)—a vacuolar membrane protein that functions as a putative Mg<sup>2+</sup> transporter and shares similarities with Alr1 and Alr2—is responsible for Mg<sup>2+</sup> efflux from vacuoles. Genetic evidence shows that this protein functions by releasing Mg<sup>2+</sup> into the cytosol under Mg<sup>2+</sup>-deficient conditions [9].

Besides vacuoles, mitochondria serve as another intracellular Mg<sup>2+</sup> pool. Two mitochondrial Mg<sup>2+</sup> transporters, Mrs2 and Lpe10, were reported to be responsible for the influx of Mg<sup>2+</sup> into mitochondria [10–13]. *MRS2* (mitochondrial RNA splicing 2) was initially identified for its requirement in mitochondrial group II intron splicing and growth on non-fermentable media [10]. This is a result of its intra-mitochondrial Mg<sup>2+</sup> deficiency affecting the ribozyme [11] because Mrs2 plays an essential role in mitochondrial Mg<sup>2+</sup> influx [12]. The decrease in mitochondrial Mg<sup>2+</sup> content of the *mrs2Δ* mutant can be

Abbreviations: FC12, Fos-choline-12; ICP-MS, inductively coupled plasma mass spectrometry; IPTG, isopropyl-β-D-thiogalactopyranoside; Mg<sup>2+</sup>, magnesium; RT-PCR, reverse transcription polymerase chain reactions

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rescued by the expression of bacterial  $Mg^{2+}$  transporter CorA fused to a mitochondrial N-terminal leader sequence of *MRS2* [13]. The functional complementation was also observed with the orthologue of *Mrs2* in humans [14].

Yeast Lpe10 also localizes at the mitochondrial inner membrane and shares 32% of its sequence identity and a conserved GMN motif with *Mrs2* [15]. Similarly, Lpe10 disruptant exhibited defects in both growth on glycerol-based media and RNA processing, together with a reduction in the mitochondrial  $Mg^{2+}$  content. Overexpression of *LPE10* leads to a sharp increase of  $Mg^{2+}$  in mitochondria, indicating its structural and functional similarities to *Mrs2* [15]. A tight interaction between Lpe10 and *Mrs2* and their hetero-oligomerization was discovered [16]. However, these two proteins cannot fully substitute for one another, indicating that their functions are not redundant. In fact, deletion of *LPE10* leads to a decrease in the mitochondrial membrane potential, which is not observed when *MRS2* is deleted [16].

To investigate whether other partners might be involved in the regulation of  $Mg^{2+}$  homeostasis in mitochondria, we arranged a genetic screening to look for the suppressors of the *mrs2Δ* mutant. Previously, we isolated a novel gene *YMR166C* as a candidate involved in mitochondrial  $Mg^{2+}$  homeostasis [17]. However, we were uncertain about how exactly *YMR166C* is involved in this process. We now show that *YMR166C* is a mitochondrial  $Mg^{2+}$  exporter. We renamed *YMR166C* to *MME1* (mitochondrial magnesium exporter 1). Mitochondrial  $Mg^{2+}$  homeostasis turns out to be a finely controlled process directed by the combinatory work of the importers and the exporter.

## 2. Materials and Methods

### 2.1. Strains, growth medium, and plasmids

Yeast transformation was carried out by the standard lithium acetate method [18]. Empty vectors were transformed in parallel to serve as negative controls. Single colonies of each transformed yeast strain were picked out and grown on synthetic selective media. Glucose (2%) was added into YPD or SD medium, while 2% glycerol was added into YPG or SG medium. The SD- $Mg^{2+}$  media used in this study were prepared with a yeast nitrogen base without the addition of  $MgSO_4$ . The SD- $Mg^{2+}$  media were carefully prepared with ultra-pure water, high-purity reagents ( $\geq 99.99\%$ ), and a clean container to avoid any  $Mg^{2+}$  contamination.

Expression plasmids used in yeast were pRS315, pGPD-413, and pTEF-425 obtained from ATCC [19]. For heterologous expression in *E. coli*, the plasmid used was a home-made pEXS-DH, generated on the backbone of pET-22b(+). The region between the T7 transcription start and T7 terminator was replaced by an N-terminal  $8 \times$  HIS tag sequence followed by a TEV cleavage site and a C-terminal  $8 \times$  HIS tag sequence. The linker sequence had been optimized for protein expression.

### 2.2. Measurement of metal content in isolated mitochondria

Isolation of yeast mitochondria was performed according to a previous protocol [20]. Protein level of each mitochondrial sample was determined by the BCA Protein Assay Kit (Thermo Scientific Pierce, Waltham, MA, USA). The content of each metal ion was determined by inductively coupled plasma mass spectrometry (ICP-MS) XII (Thermo Electron Corp., Waltham, MA, USA) by the Analysis Center of Tsinghua University.

### 2.3. Measurement of mitochondrial $Mg^{2+}$ by spectrofluorometry

Mitochondria were isolated from cells of the wild-type and *mme1Δ* mutant and then transferred into a nominally  $Mg^{2+}$ -free solution for loading of the  $Mg^{2+}$ -sensitive fluorescent dye, mag-fura-2-AM (Molecular Probe). Loading was performed by incubating mitochondria (0.5

mg/ml) in SH buffer (0.6 M sorbitol, 20 mM HEPES  $\pm$  KOH; pH = 7.4) with 5  $\mu$ M mag-fura-2-AM for 35 min at 25 °C. Mitochondria were subsequently washed three times with SH buffer to remove excess fluorescent dye. A further incubation for 35 min was carried out to allow a complete hydrolysis of the fluorescent dyes, and the mitochondria were washed twice before measuring the fluorescence intensity. To analyze  $Mg^{2+}$ -dependent changes in mitochondria, external  $Mg^{2+}$  was elevated successively to a final concentration of 10 or 20 mM. Fluorescence intensities at 340 nm and 380 nm were recorded continuously [12]. Concentrations of intra-mitochondrial  $Mg^{2+}$  were calculated from the 340/380 nm ratio according to the formula of Grynkiewicz et al. with a dissociation constant of the mag-fura-2  $\pm$   $Mg^{2+}$  complex of 1.52 in SH buffer and 1.89 in KCl buffer (150 mM KCl, 300 mM sucrose, 20 mM HEPES  $\pm$  KOH; pH = 7.4) [21].

### 2.4. Protein purification

Mme1 protein was expressed in *E. coli* CD41 (DE3) cells cultured in 4 L LB media containing ampicillin. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG, 0.5 mM) was added into the cells at an OD<sub>600</sub> of about 0.3 to induce protein expression at 16 °C for 16–20 h. The culture was then harvested and resuspended (0.1 g/ml) in buffer A (20 mM Tris, 150 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 1 mg/ml DTT, 1 mg/ml RNAase; pH = 7.5) and broken by French pressure cell at 16,000 p.s.i three times. Unbroken cells and inclusion bodies were removed by centrifugation at 24,000 g for 40 min at 4 °C. Then, the supernatant was again centrifuged with 250,000 g for 1 h and 10 min. The pellet (membrane and membrane proteins) was resuspended and dissolved with buffer B (20 mM Tris, 150 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 30 mM imidazole; pH = 7.5). FC12 was added to a final concentration of 0.15% (w/v), and the unsolubilized pellet was centrifuged at 200,000 g for 30 min at 4 °C. The supernatant containing the Mme1 protein was then purified by nickel-affinity chromatography. Briefly, the supernatant was loaded onto a 1 ml volume of pre-equilibrated Ni-NTA agarose column (Invitrogen) and washed with 30 column volumes of buffer B. The column-bound protein was eluted with 5 column volumes of buffer C (20 mM Tris, 150 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 300 mM imidazole, 0.15% FC12; pH = 7.5). To further purify the protein and dissolve the protein in buffer suitable for the following proteoliposome experiments, the eluted protein was passed through a Sephadex 200 column with buffer D (20 mM HEPES, 100 mM KCl, 15 mM  $MgCl_2$ , 0.5 mM ATP-Mg, 0.15% FC12; pH = 7.0) [22].

### 2.5. $Mg^{2+}$ transport assay of reconstituted proteoliposomes

Assays of  $Mg^{2+}$  transport activity of Mme1 in proteoliposomes were accomplished according to the published methods with some modifications [22,23]. Chloroform-methanol (3:1) was added to dissolve the yeast total lipids (Avanti Polar Lipids, Inc. Alabama, USA), and the lipids were subsequently dried in a rotary evaporator at 30 °C for another 30 min to remove the methanol and traces of chloroform. The dried lipids were suspended at room temperature to a final concentration of 10 mg/ml in buffer E (20 mM HEPES, 100 mM KCl, 15 mM  $MgCl_2$ , 0.5 mM ATP-Mg; pH = 7.0). The lipid suspension was then sonicated to generate a suspension of small unilamellar vesicles (SUVs). The SUV suspension was then flash-frozen in liquid  $N_2$  and subsequently slowly thawed at room temperature for three cycles.  $Mg^{2+}$  was loaded into the liposomes using the above steps. The liposomes were then destabilized by the addition of 0.5% (w/v) Triton X-100 and incubated for 3 h at room temperature with agitation. Afterwards, purified Mme1 was added with a lipid/protein ratio of 40:1 (w/w), and the mixture containing 0.5% (w/v) Triton X-100 was incubated for 2 h at 4 °C with agitation. The detergents were removed by incubating the mixture for 1–2 h at 4 °C with agitation with additional Bio-Beads SM-2 (Bio-Rad) at a

bead (wet weight)/detergent ratio of 60:1 (w/w). Bio-Beads SM-2 were then removed from the solution, and a second aliquot of Bio-Beads SM-2 was added. The sample was incubated for 2–3 h at 4 °C with agitation. The proteoliposome solution was separated from the Bio-Beads SM-2 and then centrifuged at 210,000 g for 30 min at 4 °C. The pellets of liposome were resuspended in buffer F (20 mM HEPES, 100 mM NaCl; pH = 6.0). To ensure more complete  $Mg^{2+}$  transport by Mme1, the suspension of liposomes was incubated at 30 °C for 2 h. Finally, the liposomes were spun down and washed with corresponding reaction buffer F twice and the  $Mg^{2+}$  contents within the liposomes were determined by ICP-MS. The influx measurements were similar with the efflux assays except that the empty proteoliposomes, without loading 15 mM  $MgCl_2$  and 0.5 mM ATP-Mg, were incubated in the reaction buffer containing 15 mM  $MgCl_2$ , with or without adding 0.5 mM ATP-Mg.

## 2.6. Statistical analysis

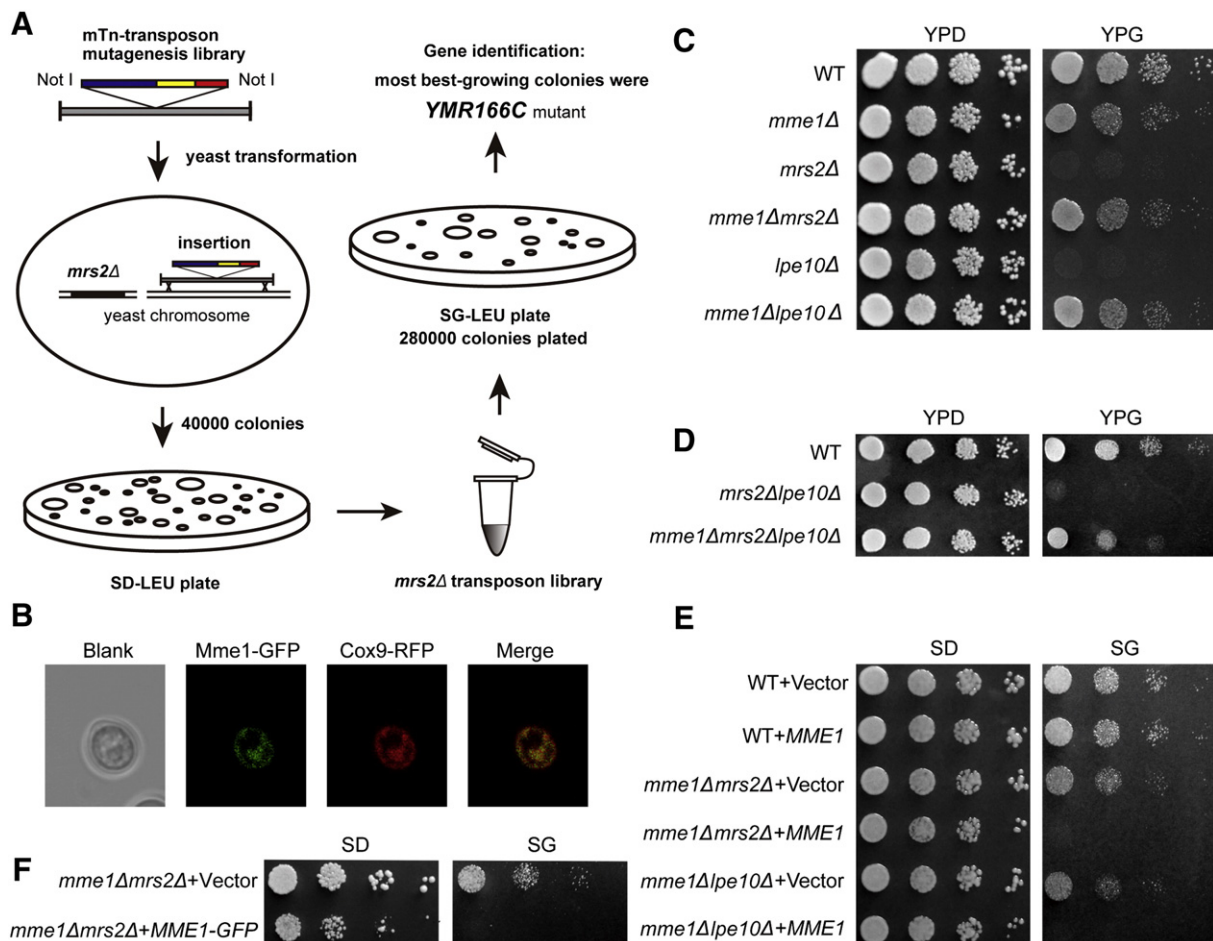
All data were analyzed by Student's *t* test. Statistical results were presented as means  $\pm$  S.D. Asterisks indicate critical levels of significance (\**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001).

## 3. Results

### 3.1. Identification of Mme1 as an antagonist of mitochondrial $Mg^{2+}$ importers Mrs2 and Lpe10

The *mrs2* $\Delta$  mutant loses the ability to respire [10]. To identify other genes involved in mitochondrial  $Mg^{2+}$  homeostasis, we previously initiated a genetic screening to search for genes whose mutations are able to suppress the growth defect of *mrs2* $\Delta$  on non-fermentable media (Fig. 1A). *YMR166C* (hereafter called *MME1*) was isolated in this process. Deletion of *MME1* is able to reverse the growth and group II RNA splicing defects in the *mrs2* $\Delta$  mutant [17].

*MME1* is a nuclear gene that encodes a protein product consisting of 368 amino acids. It is predicted to be a mitochondrial inner membrane transporter belonging to the mitochondrial carrier family [24,25]. However, little is known about the molecular function of this protein. As a first step towards the molecular characterization of Mme1, we determined its intracellular localization by confocal microscopy. As shown in Fig. 1B, the fluorescence pattern of the GFP-tagged Mme1 well overlapped that of the RFP-tagged Cox9 (subunit VIIa of cytochrome *c* oxidase [Complex IV], the terminal member of the mitochondrial inner membrane electron transport chain). This result supports that



**Fig. 1.** Mme1 antagonizes the functions of mitochondrial  $Mg^{2+}$  importers, Mrs2 and Lpe10. (A) A schematic summarizing the genetic screening process based on mTn-lacZ/LEU2 transposon insertions to identify the suppressors of *mrs2*. (B) Subcellular localization of Mme1 in the mitochondria, as indicated by the colocalization of Mme1-GFP and Cox9-RFP. GFP and RFP were integrated into the C-terminal of the endogenous chromosomal *MME1* and *COX9*, respectively. Cox9-RFP was used to indicate the mitochondria inner membrane. (C and D) Loss of *MME1* could dramatically rescue the no-growth phenotype of *lpe10* $\Delta$  and *mrs2* $\Delta$  *lpe10* $\Delta$  on glycerol medium. The *mrs2* $\Delta$  here served as a positive control. (E) Additional expression of Mme1 in the double mutant of *mme1* $\Delta$  *lpe10* $\Delta$ , as well as *mme1* $\Delta$  *mrs2* $\Delta$ , could suppress the rescue. *MME1* was cloned into the single-copy centromeric expression plasmid pRS315 with *MME1*'s original promoter and terminator to ensure the endogenous expression level. (F) Expression of Mme1-GFP in the double mutant of *mme1* $\Delta$  *mrs2* $\Delta$  could suppress the growth on glycerol medium, suggesting that the Mme1-GFP used to determine the mitochondrial membrane localization of Mme1 is functional.



Mme1 is localized in the mitochondrial membrane, consistent with the prediction based on its primary sequence.

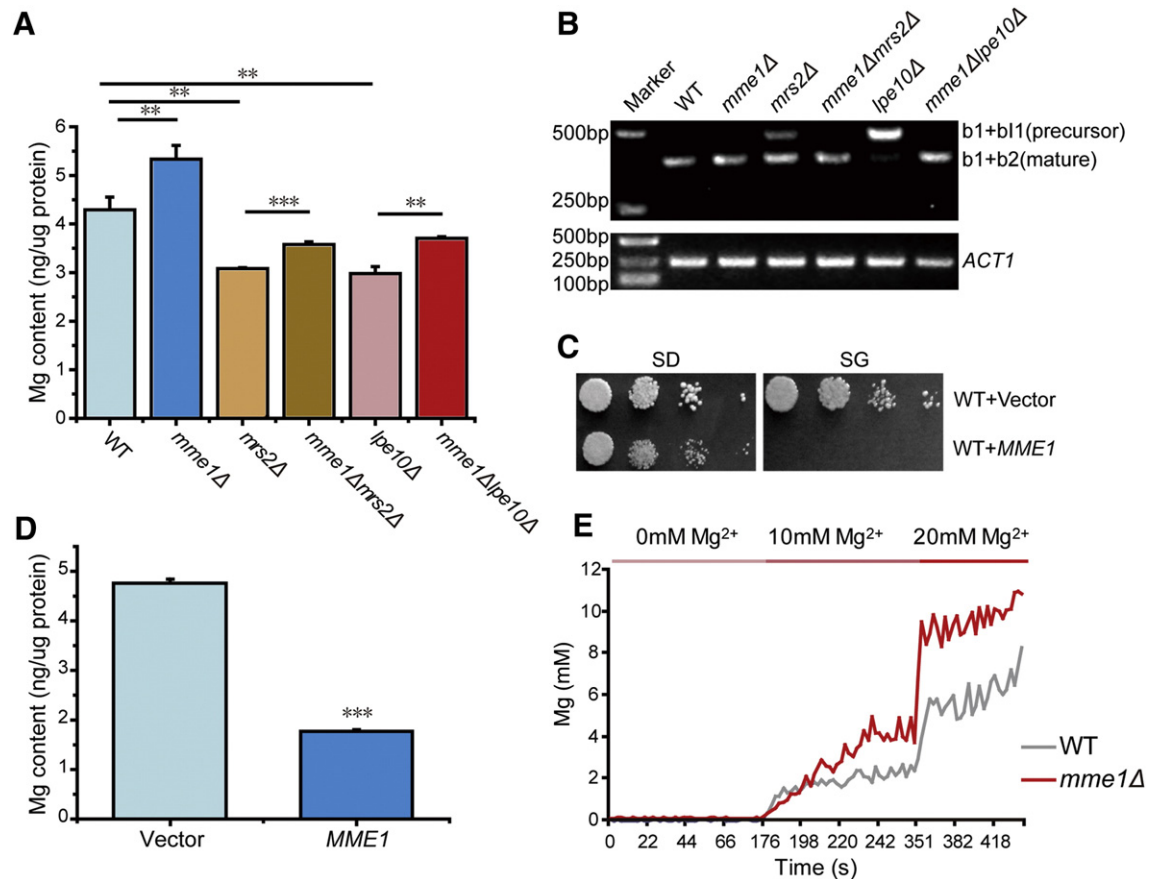
Although *MME1* was proposed as a candidate involved in mitochondrial  $Mg^{2+}$  metabolism, it was not known whether *mme1* suppresses the defects of *mrs2Δ* mutant by directly and independently regulating mitochondrial  $Mg^{2+}$  homeostasis. To explore how Mme1 might participate in mitochondrial  $Mg^{2+}$  homeostasis, we tested whether *mme1* could also be a suppressor of *lpe10* synchronously. We reasoned that if *MME1* deletion suppressed *mrs2Δ* by independently affecting mitochondrial  $Mg^{2+}$  homeostasis, it would also be able to suppress Mrs2's functional homologue Lpe10 as well. Spotting assay results showed that the deletion of the *MME1* gene could also completely rescue the growth defect of the *lpe10Δ* and *mrs2Δ lpe10Δ* mutant strains on non-fermentable carbon sources, as that of *mrs2Δ* (Fig. 1C and D). However, when an endogenous level of Mme1 was provided additionally in the *mme1Δ mrs2Δ* and *mme1Δ lpe10Δ* double mutants, the growth defect phenotype reappeared, proving that the growth of the double mutants was truly restored by the deletion of *MME1* (Fig. 1E). Similar results were also obtained when Mme1-GFP fusion protein was expressed in the *mme1Δ mrs2Δ* double mutant strain, suggesting that the Mme1-GFP fusion protein used for the above localization study is functional (Fig. 1F).

We speculated that *mme1* might suppress *mrs2* and *lpe10* through directly affecting mitochondrial  $Mg^{2+}$  levels. To test this hypothesis, the  $Mg^{2+}$  content in the mitochondria of *mme1Δ* was measured. As shown in Fig. 2A, disruption of *MME1* by itself led to a slight but

significant increase of mitochondrial  $Mg^{2+}$  level. The *lpe10Δ* mutant carried a lower amount of  $Mg^{2+}$ , and loss of *MME1* in the *lpe10Δ* mutant resulted in a significant but not yet complete restoration of the  $Mg^{2+}$  content, similar to what was observed in the *mrs2Δ* mutant strain in both this and previous studies. This result suggests that Mme1 may function directly in the regulation of steady-state concentrations of mitochondrial  $Mg^{2+}$ . The suppression effect of *mme1* on *mrs2* and *lpe10* thus can be explained by the antagonizing effects of two factors, one enhancing and one reducing the mitochondrial  $Mg^{2+}$  level.

Because mitochondrial  $Mg^{2+}$  is critical for the splicing of group II intron RNA, which includes those of mitochondrial transcripts of *COX1* and *COB* [26], we further tested whether the deletion of *MME1* could rescue the RNA splicing defect in the *lpe10Δ* mutant, as observed in the *mrs2Δ* mutant. As shown in Fig. 2B, almost all the *COB* transcripts of the wild-type yeast were in the mature form, while there were plenty of immature pre-mRNAs of *COB* in the *mrs2Δ* and *lpe10Δ* yeast cells, consistent with previous reports from others [10,15]. Deletion of *MME1* can fully rescue the group II RNA splicing defect in the *mrs2Δ* and *lpe10Δ* disruptants, as attested by a dramatic increase of mature mRNA as well as an almost complete disappearance of immature mRNA in these double mutants (Fig. 2B).

Taken together, the deletion of *MME1* can rescue various defects observed in the *lpe10Δ* disruptant as in the *mrs2Δ* mutant, likely through an independent and opposite effect on mitochondrial  $Mg^{2+}$  homeostasis. Since Mme1 is a mitochondrial carrier protein [24], we proposed that Mme1 might act as an  $Mg^{2+}$  exporter.



**Fig. 2.** Mme1 functions as a mitochondrial  $Mg^{2+}$  regulator in yeast. (A) Deletion of *MME1* could significantly restore  $Mg^{2+}$  deficiency in the mitochondria of *lpe10Δ* and *mrs2Δ* mutants.  $Mg^{2+}$  contents in the isolated mitochondria were normalized by total mitochondrial protein contents. Values are presented as mean  $\pm$  S.D.;  $n \geq 3$ . (B) Absence of *MME1* restored group II RNA splicing defects of *mrs2Δ* and *lpe10Δ* mutants. RT-PCR assays were performed with a mixture of three oligonucleotide primers matching two different exons and one intron of *COB*. PCR product of *ACT1* was used as the loading control. (C) Overexpression of *MME1* resulted in growth deficiency on non-fermentable media with glycerol as the sole carbon source. *MME1* was cloned into a GPD promoter-driven vector, pGPD-413. (D) Overexpression of *MME1* led to a dramatic decrease of the mitochondrial  $Mg^{2+}$  content. Values are presented as mean  $\pm$  S.D.;  $n \geq 3$ . (E) Response of mitochondrial  $Mg^{2+}$  levels to external  $Mg^{2+}$ . The change of  $Mg^{2+}$  was indicated by the use of a  $Mg^{2+}$ -sensitive fluorescence probe, mag-fura-2. The extra-mitochondrial  $Mg^{2+}$  concentration was stepwise raised to a final concentration of 10 mM and then to 20 mM.

### 3.2. *Mme1* overexpression leads to mitochondrial $Mg^{2+}$ reduction

If *Mme1* mediates  $Mg^{2+}$  efflux from the mitochondria while *Mrs2* and *Lpe10* are responsible for  $Mg^{2+}$  influx, the phenotype of overproducing *Mme1* would be quite similar to that of mutants lacking *Mrs2* or *Lpe10*. As predicted, yeast strain overexpressing *Mme1* showed a severe growth defect on glycerol medium similar to that of the *mrs2Δ* or *lpe10Δ* mutants (Figs. 1C and 2C).

Next, we sought to clarify whether the growth defect of the *Mme1*-overexpressing strain resulted from a decrease of mitochondrial  $Mg^{2+}$  as seen with *mrs2Δ* or *lpe10Δ* mutants. For that, the mitochondrial  $Mg^{2+}$  contents in both yeast strains transformed with an empty vector and *MME1* respectively were measured. Consistently, mitochondria of the strain overexpressing *Mme1* contained a significantly lower  $Mg^{2+}$  concentration than that of the control strain (Fig. 2D).

We then sought to measure the time-course response of *Mme1* to extra-mitochondrial  $Mg^{2+}$ , using an  $Mg^{2+}$ -sensitive fluorescent dye, mag-fura-2. Mitochondria were isolated from cells of wild-type and *MME1*-deleted yeast strains and transferred into a nominally  $Mg^{2+}$ -free solution to facilitate the loading of the membrane-permeant acetoxymethyl ester (AM) of mag-fura-2, activation of the probe, and determination of the basal intra-mitochondrial free  $Mg^{2+}$ . When the extra-mitochondrial  $Mg^{2+}$  concentration was increased stepwise to 10 and then to 20 mM, the fluorescent intensity ratio of 340/380 nm (340 and 380 nm are standard excitation wavelengths for the ion-bound and the free mag-fura-2, respectively) also immediately increased, indicating that  $Mg^{2+}$  transiently and instantly crossed the mitochondrial membrane. The increased rate of  $Mg^{2+}$  loading in the wild-type yeast is much lower than that of the *mme1Δ* mutant (Fig. 2E). We explain this by the fact that when *Mme1* was functional, some  $Mg^{2+}$  efflux occurred, resulting in a reduced mitochondrial  $Mg^{2+}$  accumulation. All these data suggest that *Mme1* works opposite to *Mrs2* and *Lpe10*, and provide further evidence supporting the notion that *Mme1* may be responsible for the efflux of mitochondrial  $Mg^{2+}$ .

### 3.3. Heterologous expression of yeast *Mme1* in *E. coli* reduces its $Mg^{2+}$ level

Mitochondria are thought to originate from proteobacteria that were taken up by a type of nucleated cell and in some aspects mimic bacterial cells such as *E. coli*. To obtain more direct and convincing evidence, the  $Mg^{2+}$  transport activity of *Mme1* was examined in a heterologous biosystem in which *Mme1* was expressed in *E. coli*.

As a membrane protein, *Mme1* is difficult to significantly express and purify *in vitro*. Initially, little expression of *Mme1* was detected in *E. coli* when a number of vectors (pET-21b, pET-28a, pGEX4T-1, pQE-80 L, and pTWIN-1) were used. Nonetheless, expression of *Mme1* was finally achieved by using the pEXS-DH plasmid vector with an N-terminal HIS-tag and *E. coli* host strain CD41 (*DE3*) (*F<sup>-</sup> ompT gal dcm lon hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*) (Fig. 3A).

Results of confocal microscopy showed that the heterogeneously expressed *Mme1* protein in *E. coli* localizes in the peripheral area (plasma membrane) of *E. coli*, coinciding with the presumed localization of *Mme1* in the mitochondrial membrane in yeast (Fig. 3B).

We next asked whether *Mme1* expression in such a foreign system could facilitate cellular  $Mg^{2+}$  efflux. Interestingly, *Mme1* expression mildly but noticeably conferred *E. coli* resistance to  $Mg^{2+}$  stress, but not to  $Zn^{2+}$  or  $Na^{+}$  (Fig. 3C). The growth differences were further confirmed by culturing the vector control and *Mme1*-expressing *E. coli* strains in corresponding liquid media. When cultured in LB medium supplemented with only IPTG or together with 2 mM  $Zn^{2+}$  and 200 mM  $Na^{+}$ , there were no significant differences between the vector control and the *Mme1*-expressing *E. coli* strain. However, the *Mme1*-expressing *E. coli* strain showed significant growth advantages over the vector control in LB medium with 200 mM  $Mg^{2+}$ , indicating that *Mme1* expression specifically confers  $Mg^{2+}$  resistance (Fig. 3D).

Further analysis of cellular  $Mg^{2+}$  contents indicated that expression of *Mme1* indeed significantly reduced the  $Mg^{2+}$  level of the cells (Fig. 3E). The reduction of  $Mg^{2+}$  contents here is not as dramatic as in Fig. 2D, likely because of the foreign or non-native nature of *Mme1* when expressed in *E. coli*. Nonetheless, a decreased  $Mg^{2+}$  concentration enables the *Mme1*-expressing *E. coli* to be resistant to  $Mg^{2+}$  excess. These results suggest that *Mme1* is necessary and sufficient in mediating mitochondrial  $Mg^{2+}$  efflux.

### 3.4. The purified *Mme1* can mediate $Mg^{2+}$ export *in vitro* when reconstituted into liposomes

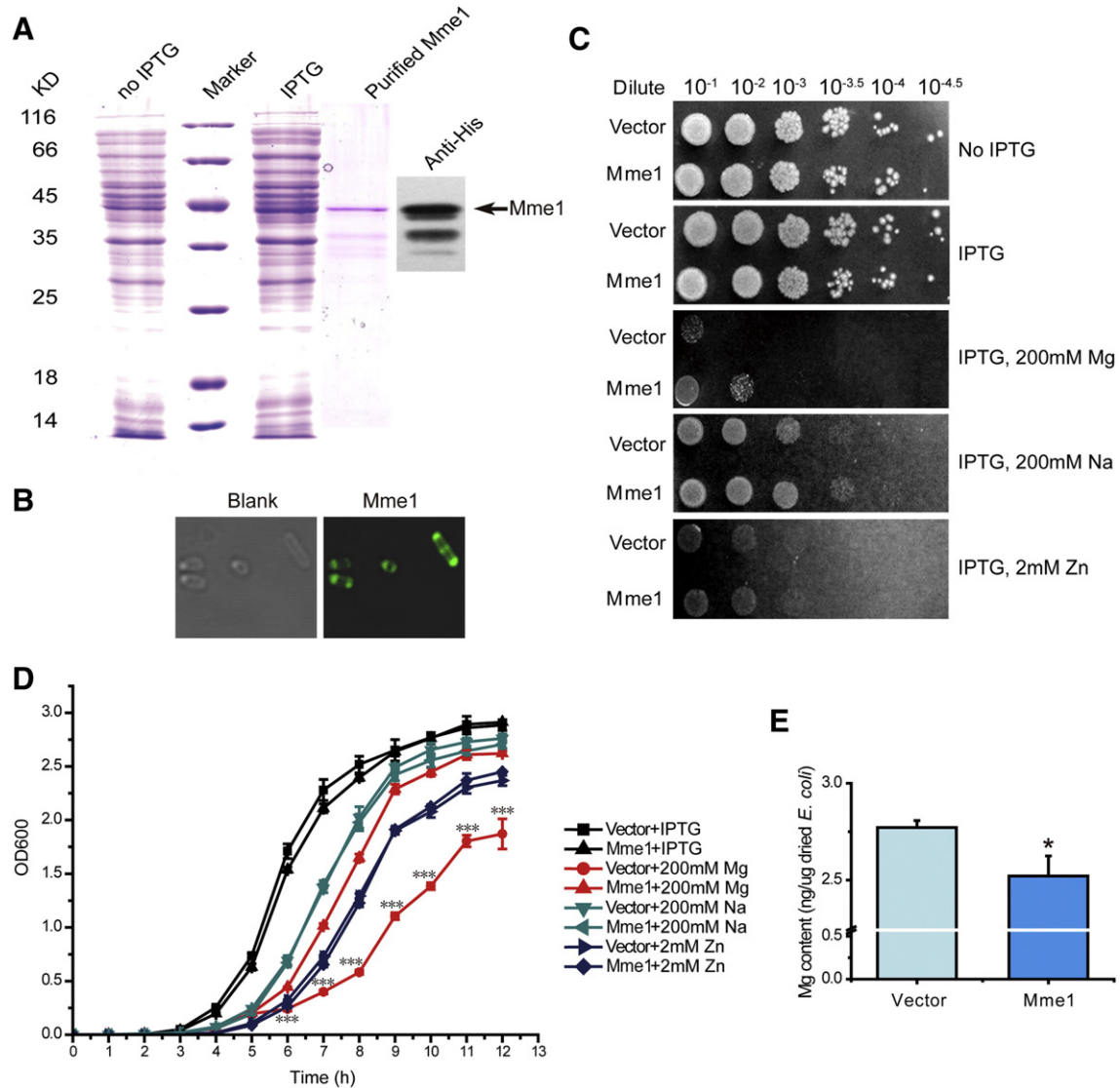
The above data together strongly suggest that *Mme1* is an  $Mg^{2+}$  exporter. Despite this, direct evidence of *Mme1* as an  $Mg^{2+}$  exporter is still lacking. One definite evidence would be a transport measurement experiment using purified *Mme1* protein performed in a clean *in vitro* system—proteoliposomes.

To obtain purified *Mme1*, we screened for detergents that might be able to dissolve it. *Mme1* was solubilized most efficiently in 2% Fos-choline-12 (FC12), better than DDM, while other detergents, including 2% CHAPS, were not very effective on *Mme1* (Table 1). *Mme1* is purified by Ni-NTA agarose column and Sephadex 200 column sequentially from total proteins (Fig. 3A) (more details in Materials and methods section).

Purified *Mme1* was then reconstituted into yeast total-lipid-based liposomes as described in the Materials and methods section. TMHMM analysis suggests that *Mme1* possesses two transmembrane domains within the 115–168 amino acid residues region. To analyze the orientation of the reconstituted *Mme1* in the proteoliposomes, they were treated separately with two proteases—trypsin and chymotrypsin. Pure recombinant *Mme1* was also subjected to the same treatments to work as controls. Protease digestion of the pure *Mme1* and proteoliposome-incorporated *Mme1* led to an almost complete disappearance of the protein band in SDS-PAGE (Fig. 4A). This result indicates that *Mme1* was preferentially inserted into proteoliposomes with most of it exposed to the exterior of the liposomes in our reconstituted samples. Were the orientation opposite, little or only part of the amino acids between the two predicted transmembrane domains of *Mme1* would have been digested. Worth noting is that this predicted orientation of *Mme1* in the membrane is opposite to that of *AtMrs2-10*, which was preferentially inserted into proteoliposomes with most of it exposed to the interior of the liposomes [22]. This difference is consistent with their opposite functions in  $Mg^{2+}$  transport with *AtMrs2-10* as  $Mg^{2+}$  importer whereas *Mme1* as a candidate of  $Mg^{2+}$  exporter.

According to this topology model, we predicted that after protease digestion, a 6 kD polypeptide of *Mme1* imbedded within the liposomes would be protected and undigested. However, we failed to detect this predicted 6 kD polypeptide band in our SDS-PAGE gel. One possible explanation for the failure might be the small size and high hydrophobicity of this protein portion. Therefore, at this moment, the proposed model remains only a favorite possibility (Fig. 4B).

The  $Mg^{2+}$  export activity of *Mme1* was subsequently measured. To ensure more complete  $Mg^{2+}$  transport, the *Mme1* proteoliposomes packed with  $Mg^{2+}$  buffer were incubated with  $Mg^{2+}$ -free reaction buffer at 30 °C for 2 h. After the reaction, the contents of the remaining  $Mg^{2+}$  within the proteoliposomes was determined by ICP-MS. The more  $Mg^{2+}$  remaining in the proteoliposomes, the less  $Mg^{2+}$  was transported out. As shown in Fig. 4C, a significant decrease in the internal  $Mg^{2+}$  content was observed when liposomes were incorporated with *Mme1*, indicating that the reconstituted *Mme1* was able to mediate  $Mg^{2+}$  efflux out of liposomes in the presence of an outward-directed  $Mg^{2+}$  gradient. Liposome integrity was intact as the  $K^{+}$  packed within the liposomes did not change appreciably (Fig. 4D). Interestingly, we observed that the  $Mg^{2+}$  export reaction occurred only in the presence of ATP-Mg packed in the proteoliposomes. In the absence of ATP,



**Fig. 3.** Mme1 regulates  $Mg^{2+}$  homeostasis when heterologously expressed in *E. coli*. (A) Coomassie brilliant blue staining result of protein samples from *MME1*-transformed *E. coli* cells with or without induction by IPTG, and purified Mme1, and purified Mme1 blotted by antibody against His tag in the N-terminal of Mme1 protein. At least some of the impurities in the purified Mme1 were due to partially degraded Mme1 as shown by Western blotting results. (B) Plasma membrane-localization of Mme1 in *E. coli*. Immunofluorescence assays were carried out in *E. coli* CD41 (DE3) cells transfected with a HIS-tagged Mme1. (C) Mme1-expressing *E. coli* cells were mildly, but selectively, resistant to  $Mg^{2+}$  when stressed by various metal ions. Spotting assays of *E. coli* cells were performed on LB medium supplemented with or without IPTG, together with 200 mM  $Mg^{2+}$ , 2 mM  $Zn^{2+}$ , and 200 mM  $Na^{+}$  respectively. A total of 200 mM  $Na^{+}$  served as an osmolarity control to exclude the possibility that Mme1-expressing *E. coli* cells were resistant to the higher osmolarity caused by 200 mM  $Mg^{2+}$  instead of to  $Mg^{2+}$  per se. (D) The growth of *E. coli* strains transformed with vector or Mme1 were confirmed in corresponding liquid medium. (E) Expression of Mme1 in *E. coli* decreased  $Mg^{2+}$  concentration within the cells. The contents of  $Mg^{2+}$  in *E. coli* cells were measured by ICP-MS and normalized by the gross dry weight of *E. coli* cells. Values are presented as mean  $\pm$  S.D.;  $n \geq 3$ .

the exporting activity almost disappeared (Fig. 4E). Therefore, it seems that ATP is indispensable for the transport of  $Mg^{2+}$  by Mme1.

We further explored the role of ATP in this process—primarily concerning whether the hydrolysis of ATP is necessary for the transport of  $Mg^{2+}$ . To address this question, ATP $\gamma$ S, a common ATP analog, which

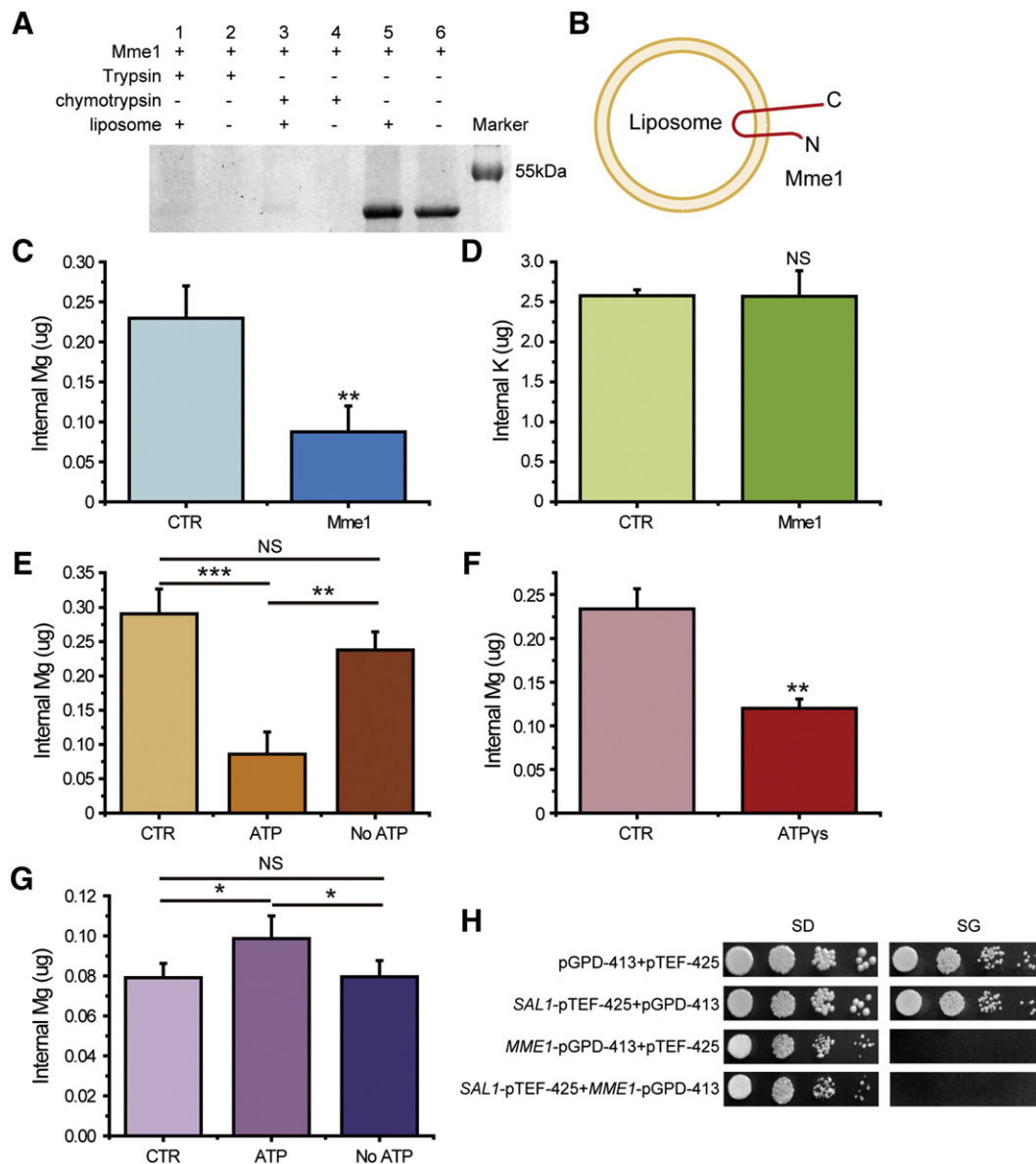
cannot be hydrolyzed, was used to replace ATP to test whether ATP $\gamma$ S can also mediate the transport. In the presence of ATP $\gamma$ S or in the absence of ATP hydrolysis, an obvious reduction, albeit less dramatic than ATP, of  $Mg^{2+}$  within proteoliposomes was also observed (Fig. 4F). This result suggests that ATP-Mg may act as a substrate for the transportation independent of ATP hydrolysis. As some structural differences between ATP and ATP $\gamma$ S exist, Mme1 may prefer the native ATP to non-native ATP $\gamma$ S in facilitating  $Mg^{2+}$  transport.

We also measured the importing activity of  $Mg^{2+}$  by Mme1 *in vitro*. The importation assay was similar to the exportation assay except that the  $Mg^{2+}$  and ATP were added to the reaction buffer instead of within the liposomes. When Mme1 was reconstituted into the liposomes, the Mme1 could also transport some  $Mg^{2+}$  into the liposomes in an ATP-dependent manner (Fig. 4G). However, the influx activity is much less robust than the efflux activity. These results suggest that Mme1 has a preference for exporting over importing  $Mg^{2+}$ .

**Table 1**  
Detergents screening for Mme1 solubilization.

Detergent	% Added	Solubilization efficiency
3-[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS)	1–2	—
Sodium cholate	1–2	—
Lauryldimethyl amine oxide (LDAO)	1–2	—
<i>n</i> -Dodecyl- $\beta$ -D-maltoside (DDM)	1–2	+
Fos-choline-12 (FC12)	1–2	++

++, high solubilization; +, low solubilization; —, little solubilization.



**Fig. 4.**  $\text{Mg}^{2+}$  efflux mediated by Mme1 in proteoliposomes *in vitro*. (A) Determination of the Mme1 orientation in proteoliposomes with protease protection assays. Protease/Mme1 ratio was 1: 50. After incubation at 30 °C for 30 min, protease digestion was terminated by the addition of SDS-PAGE sample buffer and heated for 10 min at 98 °C. The peptide that would be protected from the digestion (the two transmembrane domains plus the region in between) is smaller than 6kD (amino acid 115–168 region) and it might be too small and hard to detect here under this condition. (B) A likely representation of the topology of Mme1 in proteoliposomes. “N” and “C” stand for the N- and C-terminals of Mme1, respectively. (C)  $\text{Mg}^{2+}$  efflux mediated by Mme1-reconstituted liposomes with 0.5 mM ATP-Mg. The internal  $\text{Mg}^{2+}$  remaining in the liposomes after reactions was determined. (D)  $\text{K}^{+}$  contents within liposomes incorporated with or without Mme1. (E)  $\text{Mg}^{2+}$  transporting activities of Mme1-incorporated liposomes in the absence of ATP-Mg. (F)  $\text{Mg}^{2+}$  efflux activities of Mme1-incorporated liposomes with 0.5 mM non-hydrolysable ATP analogue ATP- $\gamma$ -S. (G)  $\text{Mg}^{2+}$  influx activities of Mme1-incorporated liposomes. Values in C, D, E, F, and G are presented as mean  $\pm$  S.D.;  $n \geq 3$ . NS stands for no significance. (H) Overexpression of SAL1, which exchanges ATP-Mg into and Pi out of mitochondria, cannot rescue the growth defect of MME1 overexpression on respiration media.

There are some other mitochondrial carrier proteins utilizing ATP-Mg as the exchanger for Pi. However, the functions of these carrier proteins are to transport adenine nucleotides across the mitochondrial inner membrane, and thus regulate the homeostasis of ATP but not  $\text{Mg}^{2+}$  within mitochondria as other ATP/ADP carriers. In yeast, Sal1 is the only ATP-Mg/Pi carrier discovered so far which transports ATP-Mg into the mitochondrial matrix and Pi out of the mitochondrial matrix [27–29]. Therefore, we wondered whether or not Sal1 functions by transporting  $\text{Mg}^{2+}$  into mitochondria, thereby working opposite to Mme1. While overexpressing Sal1 in the Mme1-overexpressing yeast strain, we found that Sal1 did not suppress the phenotype of Mme1 overexpression, indicating that Sal1 and Mme1 may not have functional interaction (Fig. 4H). The reason why overexpression of Sal1, an ATP-Mg importer, cannot rescue the phenotype of overexpression of Mme1 may

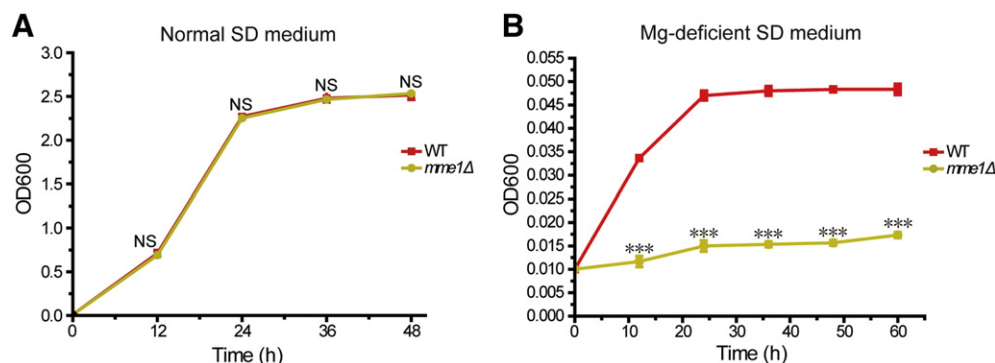
be that the accessory  $\text{Mg}^{2+}$  entering with ATP are physiologically too minor to appreciably alter the mitochondrial  $\text{Mg}^{2+}$  level.

The proteoliposome experiment provided direct evidence in supporting the hypothesis that yeast Mme1 mediates  $\text{Mg}^{2+}$  transport, mainly the efflux of  $\text{Mg}^{2+}$ . In addition, accessory proteins are not required for Mme1’s function, or in other words, Mme1 is necessary and sufficient for  $\text{Mg}^{2+}$  export.

### 3.5. Mitochondria can supplement $\text{Mg}^{2+}$ to cytoplasm for growth in $\text{Mg}^{2+}$ -depleted condition

Following the molecular characterization of Mme1 both *in vivo* and *in vitro* as described above, we then analyzed the physiological function of  $\text{Mg}^{2+}$  efflux from mitochondria mediated by Mme1. Our primary





**Fig. 5.** *MME1* provides a growth advantage under  $Mg^{2+}$ -deficient conditions. (A) Wild-type and *mme1Δ* mutant yeast strains displayed similar growth rates in normal growth conditions. Yeast was cultured in normal SD media at 30 °C and OD<sub>600</sub> measurements were carried out every 12 h. The original inoculation OD<sub>600</sub> was 0.01. (B) The growth of *mme1Δ* mutant yeast strain was hindered when transferred from normal medium to  $Mg^{2+}$ -deficient medium. The growth curves of these yeast strains in SD- $Mg^{2+}$  media (synthetic media without  $Mg^{2+}$ ) were determined as described in A. Values in A and B are presented as mean  $\pm$  S.D.;  $n \geq 3$ . NS stands for no significance.

investigation concerned whether *Mme1* contributes to the cytoplasmic  $Mg^{2+}$  levels when external  $Mg^{2+}$  is lacking. To address this concern, the growth rates of the wild-type and the *mme1Δ* mutant yeast strains were monitored. As shown in Fig. 5A, these two yeast strains exhibited identical growth rates under normal growth conditions. However, the *mme1Δ* mutant grew much worse than the wild-type control when they were transferred from normal medium to  $Mg^{2+}$ -deficient medium (Fig. 5B). This can be explained by the failure of  $Mg^{2+}$  release from the *mme1Δ* mitochondria when the cytosol is in dire need of  $Mg^{2+}$ . These data support that mitochondria are helpful  $Mg^{2+}$  pools when the cell is stressed by an acute  $Mg^{2+}$  shortage. In  $Mg^{2+}$ -deficient growth conditions, the *Mme1*-mediated release of  $Mg^{2+}$  from mitochondria into the cytosol might be important for the cell although this might compromise the mitochondria's own functions.

#### 4. Discussion

*Mme1* has been identified as the first yeast mitochondrial  $Mg^{2+}$  exporter by a genome-wide screening. Besides suppressing the growth defect in *mrs2Δ* and *lpe10Δ* mutants, deletion of *MME1* can also rescue group II RNA splicing defects and restore the decreased mitochondrial  $Mg^{2+}$  content in these two mutant strains. Further studies revealed that *Mme1* can regulate the mitochondrial  $Mg^{2+}$  efflux in yeast, mediate the  $Mg^{2+}$  transport out of the cells when heterologously expressed in *E. coli*, and display  $Mg^{2+}$  output activities when purified *Mme1* is inserted into liposomes *in vitro*, providing definite evidence that *Mme1* is a yeast mitochondrial  $Mg^{2+}$  exporter.

##### 4.1. *Mme1* as a mitochondrial $Mg^{2+}$ exporter

There are only two  $Mg^{2+}$  transport proteins that have been previously purified and reconstituted into liposomes—*T. maritima* CorA and *Arabidopsis* AtMrs2-10 [22,30]. In this work, through the use of proteoliposome, we provide compelling evidence showing *Mme1* as a mitochondrial  $Mg^{2+}$  exporter.

In the proteoliposome experiments, we found that the transport of  $Mg^{2+}$  by *Mme1* is dependent on ATP, although the hydrolysis of ATP is not required. We suspected that ATP may be transported together with  $Mg^{2+}$ . To address this possibility, we measured the efflux of ATP from ATP-Mg loaded liposomes reconstituted with *Mme1*. However, there is no obvious efflux of ATP out of the liposomes. Because the amount of ATP loaded into the liposomes is only 1/30th of that of  $Mg^{2+}$ , if the ATP is transported together with  $Mg^{2+}$  at a ratio of 1:1, we predict that only a maximum of 1/30th of the initial  $Mg^{2+}$  could be transported out. However, as shown in our results (Fig. 4C and E), ~50% of the total  $Mg^{2+}$  was transported out from the liposomes. Therefore, we suspect that the transport is mediated by an ATP/ATP-Mg

exchange. This speculated transportation mechanism requires further experimental studies to prove.

Our *in vitro* studies suggest that *Mme1* preferentially exports  $Mg^{2+}$ , though it also carries much less robust  $Mg^{2+}$  importing activity. However, the *in vivo* results indicate that *Mme1* only functions as an  $Mg^{2+}$  exporter. One possible explanation for this is that in the context of *in vivo* conditions, the net result of these transporting activities might only present as  $Mg^{2+}$  export. Therefore, it may also be the same with Mrs2. However, only the importing activity of the AtMrs2 has been measured while the exporting activity of it has not yet been measured successfully so far [22]. Another possible explanation is that *in vivo* there might be other accessory proteins interacting with *Mme1*, acting as molecular switches to control the orientation of  $Mg^{2+}$  transportation. Some other ion transporters indeed have been shown to function in this manner. For example, the central  $\alpha(1)$  pore-forming subunit (Ca(V) $\alpha(1)$ ) interacts with different accessory subunits to control the current of calcium [31]. In the case of Mrs2, Mrs2 proteins form heteromultimers with Lpe10 and work together with Lpe10 to mediate the import of  $Mg^{2+}$  into mitochondria [16]. It is possible that the interactions between Mrs2 and Lpe10 might control the orientation of  $Mg^{2+}$  transport. Alternatively, there may also be other accessory proteins to help control this process.

##### 4.2. Regulatory effects of *Mme1* on $Mg^{2+}$ homeostasis in mitochondria and cytosol

Decrease of mitochondrial  $Mg^{2+}$  in the *mrs2Δ*, *lpe10Δ* mutants or *MME1*-overexpressing strains causes growth defect on glycerol media, as a result of severe disruption of respiration because several critical enzymatic reactions in the electron transport chain require  $Mg^{2+}$  [10,11]. Additional functions modulated by  $Mg^{2+}$  within mitochondria are anion channels,  $H^+$  gradients, and membrane potentials across the mitochondrial membrane [1]. In other words, mitochondrial  $Mg^{2+}$  is involved in many processes and it is critical to maintain its proper concentration in a tight range because even a tiny alteration may affect the functions of mitochondria and even the fate of the whole cell.

As the first mitochondrial  $Mg^{2+}$  exporter identified, the role of *Mme1* on the overall control of mitochondrial  $Mg^{2+}$  must be considered in the context of the whole cell. On the one hand, perturbation of *MME1* expression corresponds to mitochondrial  $Mg^{2+}$  changes; on the other hand, isolated normal mitochondria with intact *MME1* or intact mitochondrial  $Mg^{2+}$  homeostasis systems are still sensitive to the external  $Mg^{2+}$  variation, shown by the fact that the internal  $Mg^{2+}$  concentrations fluctuate with the added  $Mg^{2+}$  levels. Therefore, mitochondrial  $Mg^{2+}$  homeostasis is likely subjected to multiple levels of control even in a unicellular eukaryotic organism. The plasma membrane-resident importers may control the amount of  $Mg^{2+}$  imported, while organelles such as the vacuole provide additional



buffers for cytosolic  $Mg^{2+}$  maintenance. On top of these controls, which already provide a relatively constant  $Mg^{2+}$  environment for the mitochondria, the importers and exporters in the mitochondria themselves then work together intricately to fine-tune the mitochondrial  $Mg^{2+}$  level. Expression perturbation of either of these partners by overexpression or removal may shift the  $Mg^{2+}$  balance. In our opinion, the exporting role of Mme1 likely acts as a second checkpoint to maintain an accurate balance of  $Mg^{2+}$  concentration within mitochondria under normal growth conditions.

On the other hand, under  $Mg^{2+}$ -deficient states, mitochondria can additionally supply some precious  $Mg^{2+}$  to the cytosol. Previous studies in mammalian cells have already uncovered a necessity of the maintenance of proper  $Mg^{2+}$  levels for the cell cycle, cell proliferation, and cell differentiation, all of which would be greatly inhibited when cellular available  $Mg^{2+}$  is limited or reduced [1]. As shown by our results in yeast, the *mme1Δ* mutants can hardly grow in  $Mg^{2+}$ -depleted fermentable medium, suggesting the significance of the contribution from the mitochondrion when external  $Mg^{2+}$  is limited. In the context of a tightly controlled mitochondrial  $Mg^{2+}$  homeostasis, we suspect that the contribution of mitochondria to cytosolic  $Mg^{2+}$  may not be too dramatic. As shown in Figs. 1C and 2A, with only a decrease of ~30% in mitochondrial  $Mg^{2+}$  content, the *mrs2Δ* and *lpe10Δ* mutants are unable to grow on glycerol medium. It can be inferred that the mitochondria must maintain a narrow range of  $Mg^{2+}$  concentrations themselves, and therefore cannot usually release too much  $Mg^{2+}$  into the cytosol without sacrificing their own functions.

## Author Contributions

Conceived and designed the experiments: YC, SZ, FS, and BZ. Performed the experiments: YC, SZ, JW, XW, BG, and QF. Analyzed the data: YC and SZ. Wrote the paper: YC, SZ, and BZ.

## Conflict of interest

The authors declare no conflict of interest.

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